ELSEVIER

Biochimica et Biophysica Acta 1512 (2001) 259–272

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.bba-direct.com

Cationic poly(ethyleneglycol) lipids incorporated into pre-formed vesicles enhance binding and uptake to BHK cells

David B. Fenske [a,*], Lorne R. Palmer [a], Tao Chen [a], Kim F. Wong [a], Pieter R. Cullis [a,b]

[a] *Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3*

[b] *Inex Pharmaceuticals Corporation, 100–8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8*

Received 5 October 2000; received in revised form 21 March 2001; accepted 27 March 2001

Abstract

This paper describes a new method for enhancing the interaction of liposomes with cells. A novel class of cationic poly(ethyleneglycol) (PEG)-lipid (CPL) conjugates have been characterized for their ability to insert into pre-formed vesicles and enhance in vitro cellular binding and uptake of neutral and sterically-stabilized liposomes. The CPLs, which consist of a distearoylphosphatidylethanolamine (DSPE) anchor, a fluorescent dansyl moiety, a heterobifunctional PEG polymer (M_r 3400), and a cationic headgroup composed of lysine derivatives, have been described previously [Bioconjug. Chem. 11 (2000) 433]. Five separate CPL, possessing 1–4 positive charges in the headgroup (referred to as CPL_1–CPL_4, respectively), were incubated (as micellar solutions) in the presence of neutral or sterically-stabilized cationic large unilamellar vesicles (LUVs), and were found to insert into the external leaflet of the LUVs in a manner dependent on temperature, time, CPL/lipid ratio, and LUV composition. For CPL/lipid molar ratios ≤ 0.1, optimal insertion levels of approximately 70% of initial CPL were obtained following 3 h at 60°C. The insertion of CPL resulted in aggregation of the LUVs, as assessed by fluorescence microscopy, which could be prevented by the presence of 40 mM Ca^{2+}. The effect of CPL-insertion on the binding of LUVs to cells was examined by fluorescence microscopy and quantified by measuring the ratio of rhodamine fluorescence to protein concentration. Neither control LUVs or LUVs containing CPL_2 displayed significant uptake by BHK cells. However, a 3-fold increase in binding was observed for LUVs possessing CPL_3, while for CPL_4-LUVs values as high as 10-fold were achieved. Interestingly, the increase in lipid uptake did not correlate with total surface charge, but rather with increased positive charge density localized at the CPL distal headgroups. These results suggest that incorporation of CPLs into existing liposomal drug delivery systems may lead to significant improvements in intracellular delivery of therapeutic agents.

Keywords: Durg delivery; Large unilamellar vesicles; Cationic lipids; Cationic PEG lipids; Liposome-cell binding

1. Introduction

Liposomes have long been recognized for their potential as in vivo drug carriers. For many conventional drugs, such as anticancer agents and antibiotics, a number of liposomal formulations have been developed which exhibit pharmacological utility [1–6]. Over the past decade, it has also become clear that liposomal systems possessing cationic lipids are

* Corresponding author. Fax: +1-604-822-4843;
E-mail: fenske@interchange.ubc.ca

0005-2736/01/$ – see front matter
PII: S0005-2736(01)00327-3

highly effective transfection agents in vitro [7,8]. The addition of cationic liposomes to plasmid DNA gives rise to large complexes (commonly known as lipoplexes) that possess excellent transfection properties in vitro, but which are ineffective in vivo due to their rapid clearance from the circulation by the lung and other first-pass organs. The need for a non-viral lipid-based system capable of systemic delivery of genes to cells led to the recent development of stabilized plasmid-lipid particles (SPLP) [9]. These particles are small ($\sim$70 nm), contain a single copy of a plasmid vector, possess long-circulation properties resulting from a surface coating of poly(ethyleneglycol) (PEG), and protect DNA from degradation by serum nucleases. Current versions of SPLP exhibit low transfection potency, however, due to low levels of accumulation into target cells [10].

In order to optimize the intracellular delivery of drugs (conventional or genetic) to cells, methods are required for increasing the uptake of liposomes into cells. Inclusion of specific targeting information on the liposome surface, such as antibody- [11–13], vitamin- [14–18], oligopeptide- [19,20], or oligosaccharide- [20] constructs specific for a particular membrane protein or receptor, have had limited success in achieving this goal in vivo, despite promising in vitro results. While specific targeting of liposomes to tissues is a desirable objective, non-specific targeting may also provide significant improvements in the effectiveness of liposomal carriers. For example, intracellular delivery of relatively specific and non-toxic genetic drugs (antisense oligonucleotides and plasmids for gene therapy) could be expected to enhance therapeutic efficacy. In this paper, we describe a non-specific targeting approach that involves increasing the electrostatic attraction between liposomes and cells by incorporation of positively-charged lipid molecules into preformed vesicles. The positively charged lipid molecules are novel structures wherein a cationic headgroup (containing between 1 and 4 positive charges) is separated from the phospholipid anchor (and thus the vesicle surface) by a flexible, hydrophilic polymer, in this case PEG. These cationic PEG-lipid (or CPL) conjugates are labeled with a dansyl group, which allows rapid and accurate quantification using fluorescence techniques. The presence of CPLs in LUVs results in dramatic increases in the binding and uptake of vesicles by BHK cells in vitro. We demonstrate the methodology for both cationic and neutral vesicles, with an emphasis on the former, as the cationic lipids present in lipoplex systems appear to play a direct role in stimulating uptake [21] and endosomal release [22] in cells. Neutral systems are also of interest as they comprise most conventional delivery systems.

The approach described herein has been developed and optimized within an in vitro context. Further development of CPL-carriers, or application of the post-insertion methodology to other targeted PEG-lipids, may lead to in vivo applications ranging from enhanced delivery of conventional drugs to gene therapy.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-phosphatidylcholine (DOPC), 1,2-dioleoyl-phosphatidylethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(Lissamine Rhodamine B Sulfonyl) (rhodamine-PE) were obtained from Avanti Polar Lipids. Cholesterol, Octyl-glucopyranoside (OGP), and HEPES were obtained from Sigma Chemical Co. DODAC and $PEGCerC_{20}$, $PEGCerC_{14}$, and $PEGCerC_8$ were generous gifts from Inex Pharmaceuticals Corp. Slide-A-Lyzer dialysis membrane cassettes were obtained from Pierce.

2.2. Design and synthesis of cationic PEG lipids

The details of the design and synthesis of the CPLs has been previously described [23]. The dansylated cationic PEG-lipid conjugates (CPLs), illustrated in Fig. 1, all have the same basic architecture, and consist of four moieties, conjugated in the following order: (i) a glycerolipid anchor (distearoylphosphatidylethanolamine (DSPE)), (ii) a lysine spacer containing a dansyl-label on the ε-amino group, (iii) a PEG_{3400} chain, at the distal end of which is covalently attached (iv) a positively charged headgroup made of linked lysine residues. By modifying the headgroup region, CPL were synthesized containing one, two, three, and four positive charges, with two varieties of the latter. As nearly all of the studies

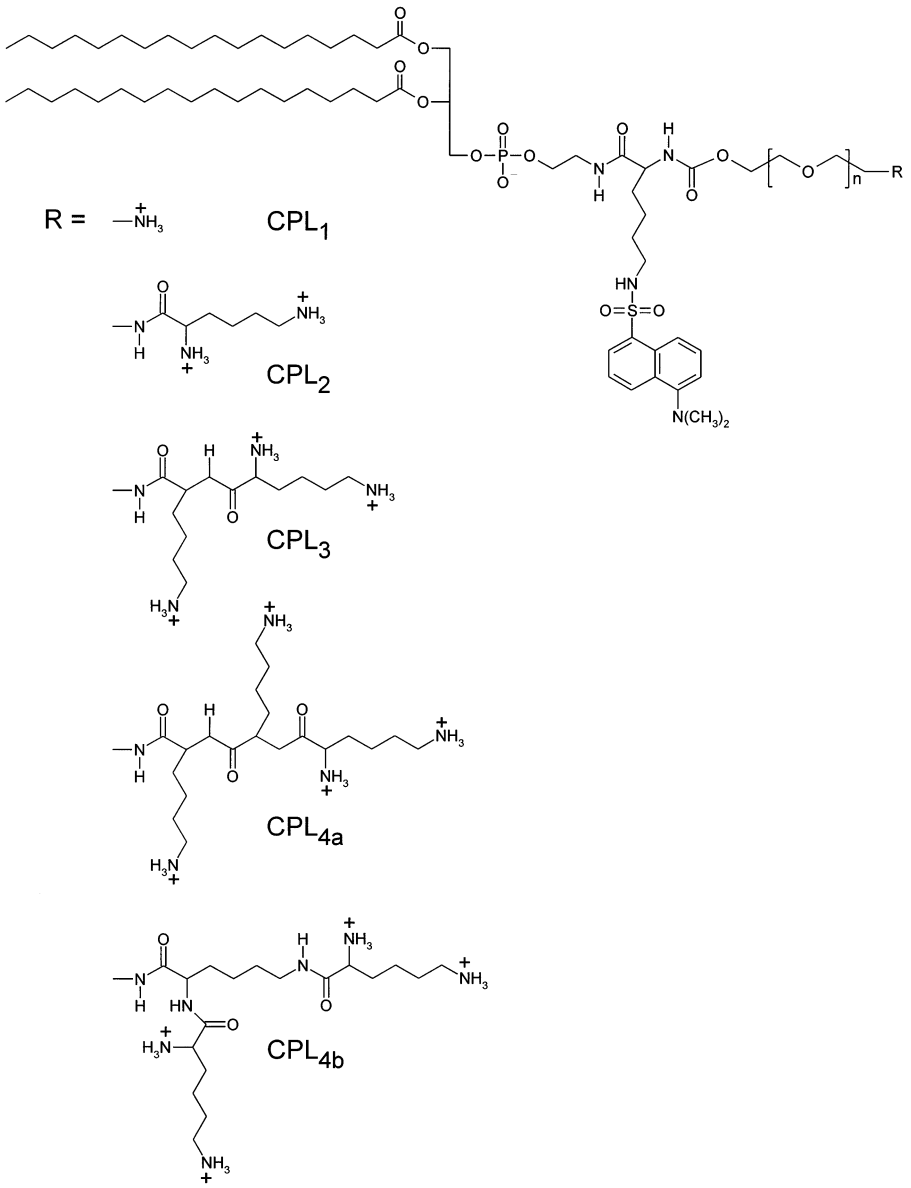

Fig. 1. Chemical structures of the DSPE-CPLs. Each CPL consists of a DSPE anchor, a dansylated lysine spacer, the PEG_{3400} spacer chain, and a cationic headgroup (R) consisting of an amino group (CPL_1), a lysine residue (CPL_2), or linked lysine derivatives (CPL_3, CPL_{4a}, CPL_{4b}). The number of the CPL refers to the number of positive charges in the headgroup at physiological pH (where each amino group will be protonated).

described in this paper involved the DSPE-CPL, the nomenclature specifies the charge of the headgroup as given in Fig. 1 (e.g., CPL_{4a}).

2.3. Preparation of vesicles

Vesicles containing DOPE (or DOPC), the cationic lipid DODAC and the stabilizing lipid $PEGCerC_{20}$ were formed using a detergent dialysis method [9]. The lipids were co-dissolved in chloroform in the appropriate ratios, following which the chloroform was removed under a stream of nitrogen and placed under high vacuum for 2 h. An aliquot of the non-ionic detergent octylglucopyranoside (OGP) (1 M in water) was then added to the dry lipid film, which was incubated for 10–20 min at 60°C with frequent

vortexing. This was followed by addition of 20 mM HEPES, 150 mM NaCl (pH 7.5), with further warming and vortexing until all the lipid was dispersed and a clear solution was obtained. For 20 mg of lipid, 0.125 ml of OGP and 1 ml of HBS were used. The lipid-detergent solutions (1–2 ml) were then transferred to Slide-A-Lyzer dialysis membranes (3 ml volume) and exhaustively dialyzed at room temperature against HBS over a period of 48 h. In general, a total volume of 8–10 l of HBS was used (4–5 changes of 2 l) for sample volumes of 1–8 ml.

Vesicles of DOPC and DOPC/Chol (55:45) were prepared by extrusion as previously described [24,25].

2.4. Insertion of CPL into preformed vesicles

The CPL were stored as micellar solutions in HBS or methanol. The CPL and the vesicles were combined to give the desired molar ratio (up to 11.6 mol% CPL relative to vesicle lipid), and incubated for a given time at the desired temperature. For most insertions, the standard conditions involved a 3 h incubation at 60°C. Following insertion, the samples were quickly cooled on ice to room temperature, and the CPL-LUV was separated from free CPL by passage down a column (1.5×15 cm) of Sepharose CL-4B equilibrated in HBS.

The insertion levels of CPL were measured by fluorescence. In all cases, the vesicles contained either 0.25 mol% or 0.5 mol% rhodamine-PE, and the CPL contained a dansyl group. After combining the CPL and lipids, a 15-μl aliquot (initial fraction) was set aside for analysis. The amount of CPL inserted into the vesicles could then be quantified by measuring the initial dansyl/rhodamine (D/R) fluorescence ratio, and the D/R ratio of the isolated CPL-LUVs. For the rhodamine assay, the excitation wavelength was 560 nm, and the emission wavelength was 590 nm. For the dansyl assay, the excitation wavelength was 340 nm, and the emission wavelength was 510 nm. In general, the excitation and emission slit widths were 10 and 20 nm, respectively. The assay was performed as follows: to an aliquot of the initial sample (2–3 μl) or the CPL-LUV (20–40 μl) was added 30 μl of 10% Triton X-100 followed by 2 ml of HBS. The fluorescence levels of both the dansyl and rhodamine labels were read consecutively using a wavelength program as per the above parameters with an emission filter of 430 nm. The %-insertion was calculated as follows:

$$\%\text{-insertion} = ([D/R]_{\text{CPL-LUV}}) \times 100/[D/R]_{\text{INITIAL}}$$

2.5. Measurement of lipid concentrations

Lipid concentrations of LUVs were measured using the standard phosphate assay [26]. Following CPL insertion, lipid concentrations were estimated for cell binding studies from the rhodamine fluorescence.

2.6. Preparation of lipid samples for high-performance liquid chromatography (HPLC) analysis

Prior to analysis by HPLC, lipids were extracted from the aqueous phase using a Bligh–Dyer procedure [27]. The organic phase was removed under a stream of N_2 gas, followed by 1 h under high vacuum. Samples were analyzed by HPLC at Northern Lipids, Vancouver, BC, Canada.

2.7. Uptake of CPL-containing LUVs by BHK cells

Approximately 10^5 BHK cells were incubated in PBS/CMG medium with 20 nmol of DOPE/DODAC/PEGCerC_{20} (84:6:10) LUVs containing either (1) no CPL, (2) 5.6% CPL_2, (3) 5.7% CPL_3, or (4) 3.4% CPL_{4b}. Incubations were performed for 1, 2, 4, and 6 h at 4°C and 37°C, the former giving an estimate of cell binding, and the latter of binding and uptake. By taking the difference of the two values, an estimate of lipid uptake at 37°C was obtained. For each timepoint, the cells were washed twice with PBS and then lysed with 600 μl of phosphate buffer (pH 8.0) containing 0.1% Triton X-100 followed by lipid and protein assays. Lipid concentrations were measured using rhodamine fluorescence, while protein was determined using the BCA assay kit obtained from Pierce. Where indicated, fluorescence micrographs were taken on an Axiovert 100 Zeiss fluorescence microscope (Carl Zeiss Jena) using a rhodamine filter from Omega Opticals (Brattleboro, VT) with the following specifications: $\lambda_{ex} = 560 \pm 20$ nm, LP = 600 nm, and DC = 590 nm.

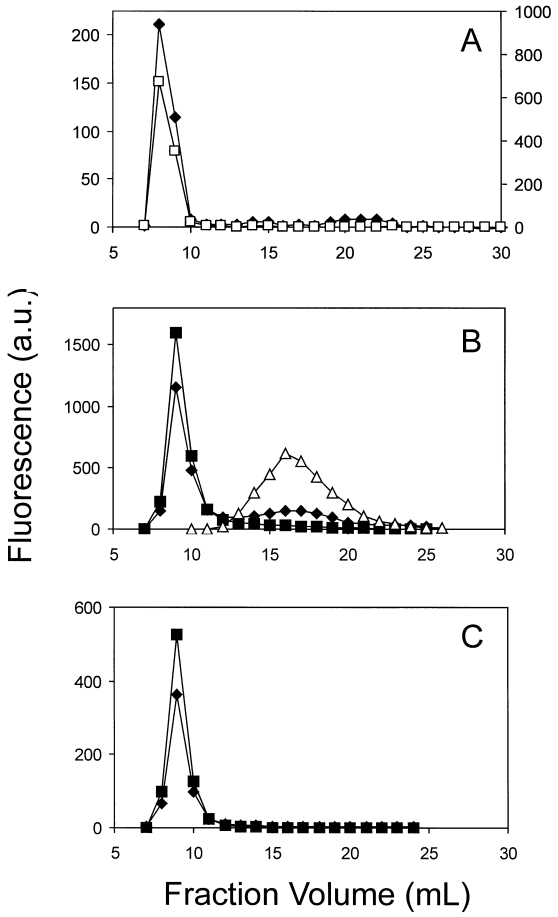

Fig. 2. (A) Insertion of CPL_{4b} into DOPC LUVs (100 nm). DOPC LUVs (2.5 μmol lipid) were incubated with 0.085 μmol CPL_{4b} (total volume 240 μl) at 60°C for 3 h, following which the sample was applied to a column of Sepharose CL-4B equilibrated in HEPES-buffered saline. One-ml fractions were collected and assayed for dansyl-labeled CPL (♦) and rhodamine-PE (□) as described. (B) Insertion of CPL_{4b} into LUVs (100 nm) composed of DOPE/DODAC/$PEGCerC_{20}$ (84:6:10). LUVs (5 μmol lipid) were incubated with 0.355 μmol CPL_{4b} (total volume 519 μl) at 60°C for 3 h, following which the sample was applied to a Sepharose CL-4B column equilibrated in HEPES-buffered saline. One-ml fractions were collected and assayed for dansyl-labeled CPL (♦) and rhodamine-PE (□) as described. The elution of free CPL (△) is also shown, demonstrating a straightforward method for isolation of the CPL-LUV. (C) Retention of CPL_{4b} in LUVs (100 nm) composed of DOPE/DODAC/$PEGCerC_{20}$ (84:6:10). The main LUV fraction from B was re-applied to a column of Sepharose CL-4B equilibrated in HEPES-buffered saline. One-ml fractions were collected and assayed for dansyl-labeled CPL (♦) and rhodamine-PE (■) as described.

3. Results

3.1. Development of insertion protocol

The transfer of PEG-lipid monomers from micelles to bilayers via the aqueous phase is a well-known phenomenon [20,28–30]. For example, $MPEG_{1900}$-DSPE was found to insert into vesicles composed of PC/cholesterol/PG to levels as high as 3 mol% [30]. As the DSPE-CPLs are structurally similar and form micellar solutions, we anticipated that incubation of CPL solutions with vesicles would result in incorporation of some CPL in the external vesicle monolayer. The sections below describe the optimization of several variables that affect the insertion process.

3.1.1. CPL insertion

The insertion of CPL_{4b} into neutral 100 nm vesicles composed of DOPC is illustrated in Fig. 2A; experimental details are given in the figure legend. The co-elution of the dansyl and rhodamine labels on a Sepharose CL-4B column demonstrates incorporation of the CPL in the LUVs. In this case, 84% of the CPL was incorporated into the LUVs, corresponding to a final insertion of 2.9 mol%, and thus only a trace of free CPL is observed trailing the CPL-LUV fractions. This is more clearly seen in Fig. 2B, where CPL_{4b} has been inserted into more complex positively charged vesicles composed of DOPE/DODAC/$PEGCerC_{20}$ (84:6:10). Here, the co-elution of the two fluorescent labels at approx. 9 ml demonstrates 70% insertion of the CPL into the vesicles, corresponding to a final CPL concentration of 4.9 mol%. The free CPL micelles elute in a broad peak centered at 16 ml, which is separate from the vesicle peak, allowing for easy isolation of the CPL-LUV. Once inserted, the CPL_{4b} is retained and does not exchange out of the vesicles. The CPL-LUV fraction from Fig. 2B was re-eluted on the column of Sepharose CL-4B. As shown in Fig. 2C, all of the CPL remains with the LUVs.

3.1.2. Effect of time, temperature, and CPL/lipid ratio on insertion

The effects of incubation temperature and time on the insertion process are shown in Fig. 3. CPL_{4a} was incubated in the presence of DOPE/DODAC/

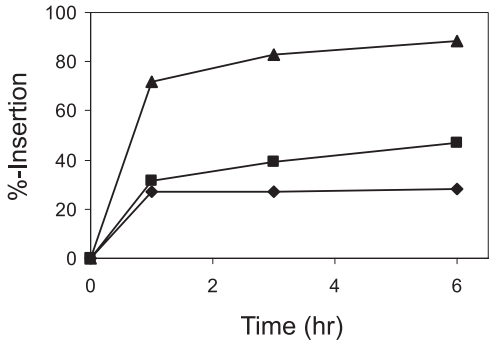

Fig. 3. Effect of time and temperature on the insertion of CPL_{4a} into DOPE/DODAC/$PEGCerC_{20}$ LUVs. Insertions were performed at 60°C (▲), 40°C (■), and 25°C (♦). For each of the three temperatures, 3 μmol lipid was combined with 0.13 μmol CPL (total volume 240 μl). At 1, 3, and 6 h, 1 μmol of lipid was withdrawn and cooled on ice to halt insertion of CPL. The samples were passed down a Sepharose CL-4B column to remove excess CPL, and assayed for CPL insertion.

$PEGCerC_{20}$ (84:6:10) at room temperature (25°C), 40°C, and 60°C, with aliquots withdrawn at 1, 3, and 6 h. The highest insertion levels were achieved at 60°C, which was therefore used in subsequent insertions. Although slightly higher insertion was obtained at 6 h, we chose 3 h to minimize potential sample degradation.

Aside from time and temperature, the parameter that will have the greatest influence on final CPL insertion levels is the initial CPL/lipid ratio. Assuming about 70% insertion, a series of incubations were performed with CPL/lipid ratios varying between 0.011 to 0.14, with the aim to achieve CPL-LUVs containing 1, 2, 4, 6, 8, and 12 mol% CPL. These results are shown in Fig. 4A, where it is seen that the insertion level remains close to 70% up to an initial CPL/lipid ratio of 0.095, above which it drops to 50% for CPL/lipid = 0.14. Similar results were obtained for other vesicle systems, including DOPE/DODAC/$PEGCerC_{14}$ and DOPE/DODAC/$PEGCerC_8$ (Fig. 4B). In general, the insertion levels obtained with DODAC-containing samples fell in the range of 65–82% for initial CPL/lipid < 0.1. The insertion levels tend to be higher at lower CPL/lipid ratios (Fig. 4B).

3.1.3. *Effect of lipid composition on insertion*

In order to see whether the insertion levels were reduced in the absence of cationic lipid, several experiments were performed on neutral vesicles containing DOPC. The compositions examined were: (A) DOPC, (B) DOPC/Chol, (C) DOPC/$PEGCerC_{20}$, and (D) DOPC/Chol/$PEGCerC_{20}$. The results, shown in Fig. 5, reveal significant levels of incorporation of both CPLs, albeit lower for CPL_{4a} (45–65%) than for CPL_{4b} (70–84%). The differential behavior observed for these two lipids is probably a reflection of the higher initial CPL/lipid ratio of the former. Nevertheless, comparison of the CPL_{4a} neutral lipid results with similar studies involving DOPE/DODAC/$PEGCerC_{20}$ reveal significantly reduced insertion in the neutral LUVs. This may be due to reduced attraction between the negatively-charged DSPE anchor and the membrane surface. Regardless, the results demonstrate that significant insertion can be achieved for both neutral and positive vesicles.

A large number of insertions have been performed using other CPLs in addition to the CPL_{4b} and

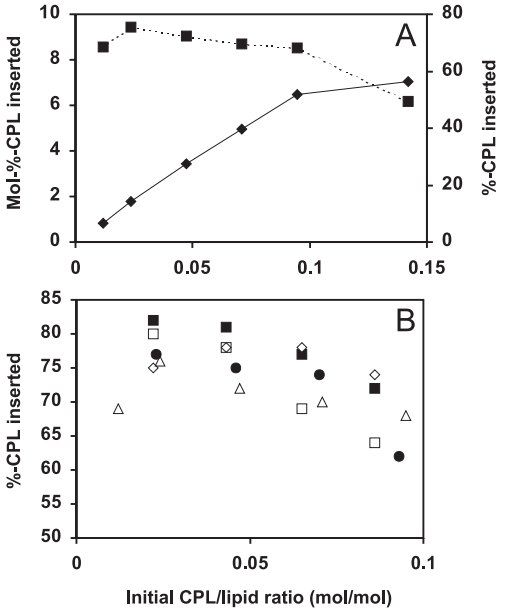

Fig. 4. (A) Effect of initial CPL/lipid ratio on final CPL_{4b} insertion levels in LUVs composed of DOPE/DODAC/$PEGCerC_{20}$ (84:6:10). Initial CPL/lipid ratios were 0.011, 0.024, 0.047, 0.071, 0.095, and 0.14. Final mol% inserted (♦): 0.8, 1.8, 3.4, 5.0, 6.5, and 7.0. The right-hand axis represents percent insertion (■). See text for further details. (B) Effect of initial CPL/lipid ratio on the %-CPL inserted into several representative LUV formulations. (□) CPL_{4a} inserted into DOPE/DODAC/$PEGCerC_{20}$ (84:6:10); (■) CPL_{4a} inserted into DOPE/DODAC/$PEGCerC_{14}$ (84:6:10); (◇) CPL_{4a} inserted into DOPE/DODAC/$PEGCerC_8$ (79:6:15); (△) CPL_{4b} inserted into DOPE/DODAC/$PEGCerC_{20}$ (84:6:10); (●) CPL_3 inserted into DOPE/DODAC/$PEGCerC_{20}$ (84:6:10).

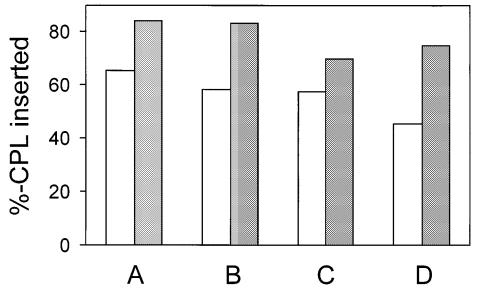

Fig. 5. Insertion of CPL_{4a} and CPL_{4b} into neutral vesicles. The initial CPL/lipid ratio was 0.065 for the CPL_{4a} (open bars) (2.5 μmol lipid and 0.21 μmol CPL) and 0.034 for the CPL_{4b} (shaded bars). Samples were incubated at 60°C for 3 h. The DOPC and DOPC/Chol LUVs were prepared by extrusion, while the others were prepared by detergent dialysis. Sample compositions: (A) DOPC, (B) DOPC/Chol (55:45), (C) DOPC/$PEGCerC_{20}$ (90:10), (D) DOPC/Chol/$PEGCerC_{20}$ (45:45:10).

CPL_{4a} (for some representative results, see Fig. 4B). Although there are some composition-dependent effects, in general we observe between 60–80% insertion for initial CPL/lipid ratios < 0.1, with higher insertion observed at lower CPL/lipid ratios. Some minor trends are worth noting. The quantity of PEG-Cer present in the LUV affects the final insertion levels. As the $PEGCerC_{20}$ content was increased from 4 to 10 mol%, the insertion level of CPL_{4b} fell from 71% to 62% (not shown).

3.1.4. Effect of CPL insertion on LUV lipid composition

The insertion of up to 7 mol% CPL into the external leaflet of an LUV might be expected to have some effect on vesicle structure or composition. Of special concern was the possibility that incorporation of CPL might lead to expulsion of some $PEGCerC_{20}$, which exchanges out of eggPC vesicles with a half-life for dissociation > 13 days [9]. This was examined by measuring the lipid composition of DOPE/DODAC/$PEGCerC_{20}$ LUVs by HPLC before and after the insertion of CPL_{4b}. As shown in Table 1, the insertion of 3.5 and 6 mol% CPL_{4b} had no significant effect on lipid composition, other than a small decrease in $PEGCerC_{20}$ content (from 8.3 to 7.5 mol%, relative to total lipid). It should be noted that the presence of 40 mM Ca^{2+}, which prevents or reverses vesicle aggregation (see Section 3.1.5), had no effect on CPL insertion levels (Table 2) or vesicle composition (Table 1).

Table 2
Effect of calcium on the insertion of CPL_{4b} into DOPE/DODAC/$PEGCerC_{20}$ (83.5:6:10) LUVs

$[Ca^{2+}]$ (mM)	Initial CPL/lipid	% Insertion	Mol% CPL inserted
0[a]	0.082	70	5.7
0[a]	0.082	75	6.1
40[b]	0.082	72	5.9
40[c]	0.082	71	5.8
40	0.049	74	3.6

[a]Results from two separate vesicle preparations.
[b]Calcium was present during insertion and removal of excess CPL on Sepharose CL-4B.
[c]Calcium was added following CPL removal on Sepharose CL-4B.

3.1.5. Effect of CPL insertion on vesicle aggregation

When examined under the fluorescence microscope, DOPE/DODAC/$PEGCerC_{20}$ LUVs labeled with 0.5 mol% rhodamine-PE exhibit a diffuse red background fluorescence. Following insertion of CPL_{4b}, large aggregates can be observed (data not shown). This aggregation can be reversed by the addition of 40 mM Ca^{2+}, following which a diffuse red

Table 1
Effect of insertion of CPL_{4b} into DOPE/DODAC/$PEGCerC_{20}$ (84:6:10) LUVs on vesicle composition

Mol% CPL_{4b}[a]	Lipid composition determined from HPLC (mol%)			
	DOPE	DODAC	$PEGCerC_{20}$	DOPE/$PEGCerC_{20}$[b]
0	83.5 ± 0.1	8.3 ± 0.2	8.3 ± 0.1	10.1 ± 0.1
3.5	84.1 ± 0.2	8.3 ± 0.01	7.5 ± 0.2	11.2 ± 0.2
5.7	84.3 ± 1.0	8.2 ± 0.1	7.5 ± 0.1	11.2 ± 0.3
5.9[c]	83.9 ± 0.6	8.4 ± 0.01	7.7 ± 0.2	10.9 ± 0.3

[a]Mol% insertion determined from dansyl/rhodamine fluorescence ratio.
[b]Molar ratio.
[c]Insertion and isolation were performed in the presence of 40 mM Ca^{2+}.

fluorescence is again observed with no aggregates. The presence of calcium prevents the formation of aggregates. Interestingly, neither the CPL insertion levels (Table 2), the composition of the CPL-LUVs (Table 1), nor the diameters of the CPL-LUVs (Table 3) are altered by the presence of calcium.

Quasi-elastic light scattering (QELS) was used to examine the effect of CPL insertion on particle diameter (Table 3). In general, the insertion of CPL led to a small increase in vesicle diameter, usually on the order of 15 nm. Thus, the diameter ± S.D. of DOPE/DODAC/PEG-Cer-C_{20} vesicles were found to increase from 70 ± 22 nm to 87 ± 24 nm upon insertion of 6 mol% CPL_{4b}. Essentially identical results were obtained in the presence of 40 mM Ca^{2+}. That the aggregation observed by fluorescence microscopy was not manifest suggests that the aggregates were too large to be measured by QELS. The small observed size increase may result from the presence of the longer CPL PEG chains [30], which may be expected to increase the particle radius. The size increase upon insertion of CPL is unaffected by the quantity of CPL inserted or by the quantity of DODAC present in the vesicles. Identical size increases were observed for CPL concentrations ranging between 1.8 and 7.0 mol% (not shown). Furthermore, increasing the DODAC content from 6% to 18% had little effect on CPL insertion or on the size increase. In all cases, samples remained optically clear, and there was no evidence of precipitation.

Table 3
Effect of insertion of CPL_{4b} into DOPE/DODAC/$PEGCerC_{20}$ (84:6:10) LUVs on vesicle diameter

Lipid composition of DOPE/DODAC/$PEGCerC_{20}$	Mol% CPL inserted	[Ca^{2+}] (mM)	Diameter ± S.D. (nm)
83.5:6:10	0	0	70 ± 22
	0	40	72 ± 22
	6.1	0	87 ± 24
	5.8	40	88 ± 25
83.5:6:10	0	0	60 ± 17
	5.5	0	75 ± 19
77.5:12:10	0	0	64 ± 18
	6.3	0	82 ± 23
71.5:18:10	0	0	67 ± 21
	6.2	0	80 ± 22

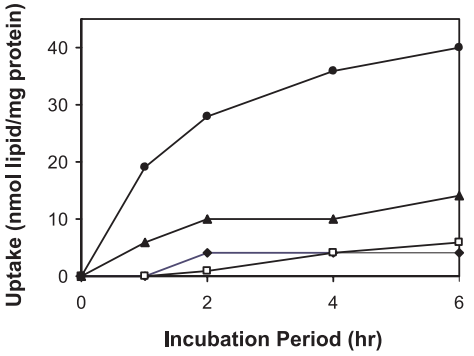

Fig. 6. Uptake of CPL-LUVs incubated in PBS/CMG on BHK cells. Approximately 10^5 BHK cells were incubated with 20 nmol of DOPE/DODAC/$PEGCerC_{20}$ (84:6:10) LUVs containing (1) no CPL (♦), (2) 5.6% CPL_2 (□), (3) 5.7% CPL_3 (▲), and (4) 3.4% CPL_{4b} (●). Incubations were performed at 4°C and 37°C, the former giving an estimate of cell binding, and the latter of binding and uptake. By taking the difference of the two values, an estimate of lipid uptake at 37°C was obtained.

3.2. In vitro uptake of CPL-vesicles

As described in Section 2, estimates were obtained for the uptake of various CPL-LUVs on BHK cells incubated on PBS/CMG. The data, shown in Fig. 6, reveals that the presence of positive charge on the CPLs can lead to significant enhancement in uptake by BHK cells. LUVs composed of DOPE/DODAC/$PEGCerC_{20}$ (and thus exhibiting a net positive charge) showed little uptake on the BHK cells. LUVs containing 5.6 mol% of CPL_2 showed similar low uptake values. Uptake was only slightly increased by the presence of 5.7 mol% of CPL_3. However, a significant increase in uptake (about 10-fold at 6 h) was realized for CPL_{4b} present at only 3.4 mol%. These results mirror our earlier studies in which CPL_{4b}-containing LUVs of DSPE/Chol/Rho-PE exhibited 50-fold increased binding and uptake on BHK cells [23].

The dramatic effect of CPL insertion on LUV binding to BHK cells is most clearly visualized using fluorescence microscopy (Fig. 7). In the absence of CPL, vesicles composed of DOPE/DODAC/$PEGCerC_{20}$ and containing a trace of rhodamine-PE exhibit little binding to cells (Fig. 7A). Incorporation of 3 mol% CPL_{4b} leads to high levels of vesicle binding and uptake (Fig. 7B). Although much of the lipid appears to be binding to the cell surface, some small punctate structures can be seen, indicating that

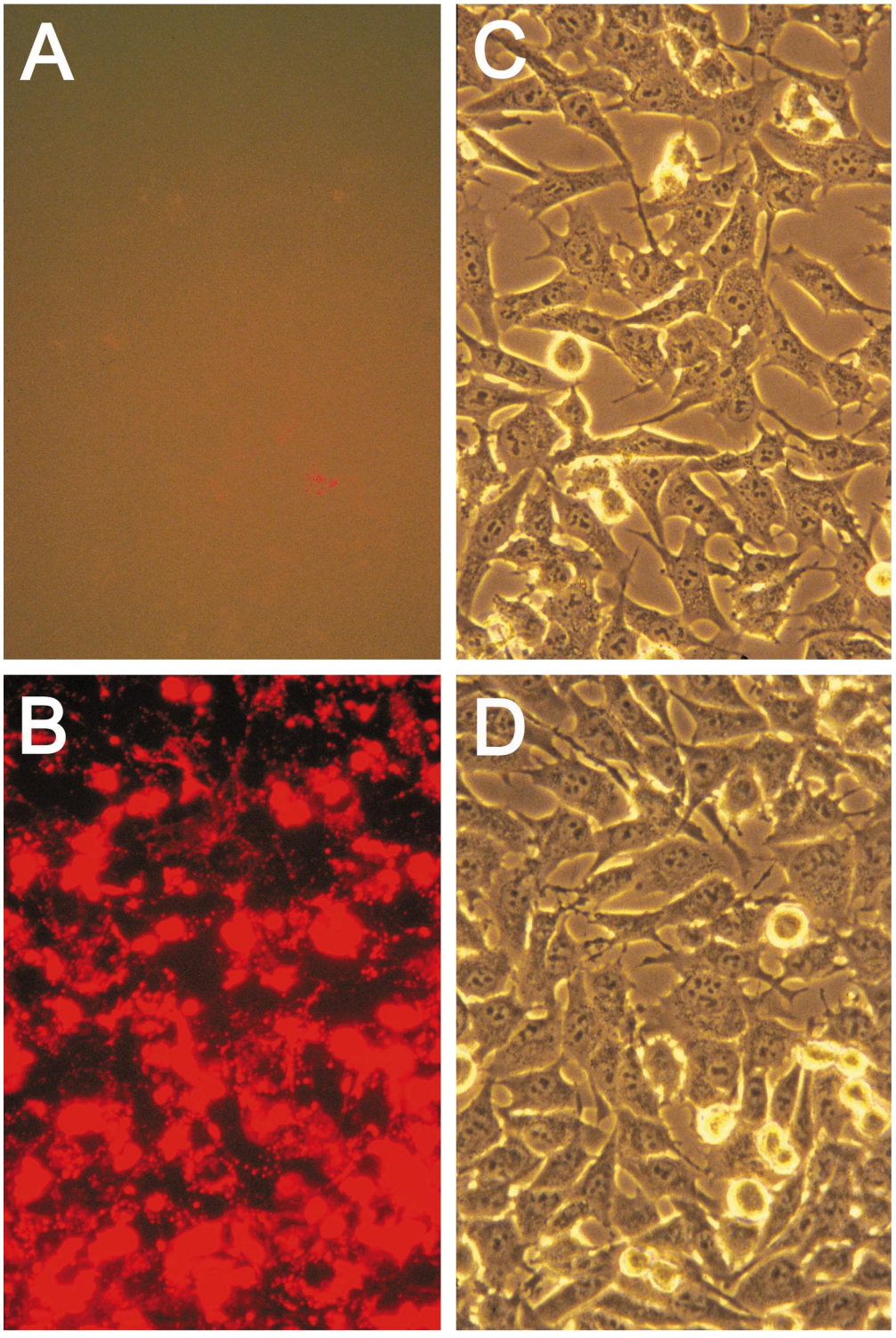

Fig. 7. (A) Fluorescence micrograph of BHK cells following a 6-h incubation in the presence of DOPE/DODAC/PEGCerC_{20} vesicles containing 0.5% rhodamine-PE. (B) As in A, except the vesicles contain 3 mol% of CPL_{4b}. (C,D) Phase contrast micrographs of the cells in A and B, respectively.

uptake of vesicles is also occurring, as shown in Fig. 6. An important point to note is that the cells (Fig. 7C,D) appear healthy following incubation in the presence of the CPL-LUVs.

Similar results to those described above have been observed for BHK cells grown in the presence of 10% FBS, for binding studies involving DOPE/DODAC/PEGCerC$_{20}$/Rho-PE LUVs containing both 3.2 mol% CPL$_{4b}$ and remote-loaded doxorubicin (drug-to-lipid ratio = 0.12 mol:mol) (D.B. Fenske, A.L. Ng, unpublished results). In addition, 10% FBS was also present in our earlier studies that demonstrated enhanced binding of CPL$_{4b}$/DSPE/Chol/Rho-PE LUVs on BHK cells [23].

4. Discussion

One of the major remaining hurdles in liposomal drug delivery is the problem of how to ensure that the contents of a carrier system are taken up and utilized by a specific target cell. Cellular uptake of liposomes involves adsorption or binding at the cell surface, followed by endocytosis. Thus factors which interfere with cellular binding will lead to low levels of intracellular delivery. This is of particular importance for 'stealth' or long-circulating liposomes that are coated with a surface layer of a hydrophilic polymer such as PEG. The very characteristic of the PEG coating which imparts long-circulation lifetimes – the formation of a steric barrier that prevents interaction with serum proteins will also minimize interactions with cells. On the other hand, factors that enhance surface binding may be expected to lead to increased cellular uptake. One approach involves attaching molecules specific for membrane receptors to liposomal surfaces. Possible candidates include oligopeptides [19,20], oligosaccharides [20], folate [14–16,18], riboflavin [17], or antibodies [11–13]. An alternate approach is to modify the charge characteristics of the liposome. It is well known that inclusion of either negative [21,31–33] or positive [21] charges in liposomes can lead to enhanced cellular uptake in vitro. Cationic DNA–lipid complexes, which are positively charged, efficient in vitro transfection agents [7,8,12,34,35], are taken up via endocytosis.

This paper describes a new approach for enhancing the interaction of liposomes with cells, a necessary step in the eventual development of non-viral systems capable of intracellular delivery. The approach involves the insertion of novel cationic PEG-lipids into pre-formed liposomes, leading to a cationic vesicle in which the positive charge involved in cell interaction is located some distance away from the vesicle surface. The process is illustrated in Fig. 8 for the insertion of a CPL$_4$ into sterically-stabilized LUVs composed of DOPE, the cationic lipid DODAC, and PEGCerC$_{20}$. This lipid composition was chosen for study for two reasons: first, it allows for efficient entrapment of plasmid DNA within small vesicular particles (SPLP) by virtue of the presence of positively charged DODAC [9], and thus has potential as a systemic gene delivery system (see below). Secondly, this composition is representative of the many sterically-stabilized drug delivery systems which contain PEG-lipids. Unfortunately, both SPLPs and the vesicles examined in the present study exhibit low levels of uptake in vitro. Insertion of CPLs leads to localization of positive charge above the surface PEG layer, thereby allowing electrostatic interactions between the CPLs and cell surfaces. For in vitro studies, this should lead to increased cellular interactions for both conventional- and PEG-containing liposomes.

One might question the advantages of inserting CPLs into pre-formed vesicles rather than simply including the CPL as a lipid component during vesicle formation. While the latter approach would work with conventional vesicles, problems would arise in formulation processes where plasmid DNA was present, such as in the formation of SPLP. Encapsulation of DNA within SPLP requires interactions between the cationic lipid DODAC and negatively charged plasmid: if present, CPL would doubtless interfere with this process, either by interacting with plasmid (and thereby reducing the CPL quantity present on the vesicle surface), or by altering the optimal charge ratio for DNA encapsulation. The post-insertion protocol has the advantage of being completely general to any liposomal system.

The approach described in this paper for inserting CPLs into pre-formed vesicles builds on several recent related studies. The transfer of PEG-lipid conjugates from micellar phase to vesicles has been described for MPEG$_{1900}$-DSPE [30], PEG$_{5000}$-DPPE and PEG$_{5000}$-DSPE [36], and for oligopeptide PEG-

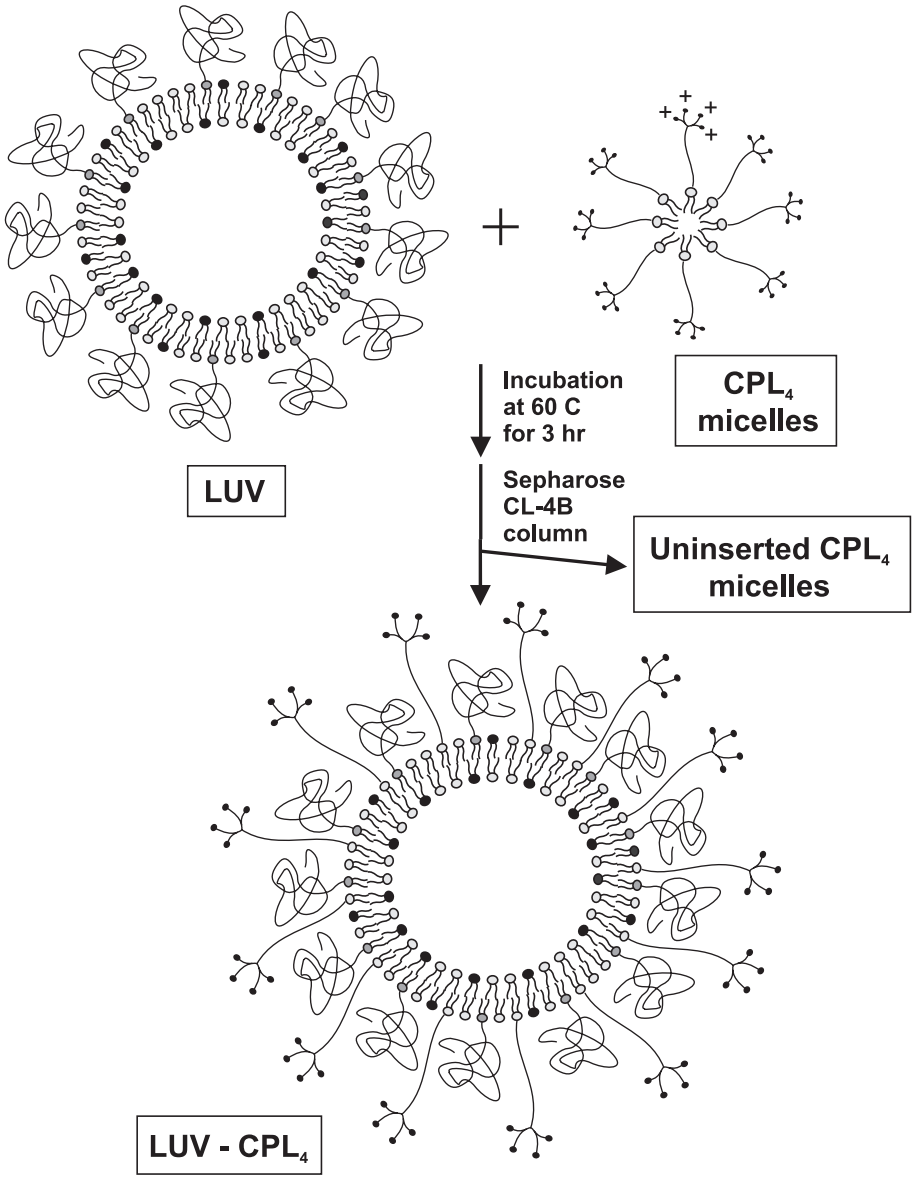

Fig. 8. Diagrammatic representation of the insertion of a CPL_4 into sterically-stabilized LUVs composed of DOPE/DODAC/$PEGCerC_{20}$ (top). Incubation of the LUVs in the presence of CPL micelles results in the insertion of some CPL into the external leaflet of the LUV, giving rise to the structure shown at the bottom. Each black dot on the CPL represents a positive charge. The key feature of the model lies in the ability to place external positive charge at some distance from the vesicle surface. The positive charges on the DODAC (black shaded lipids) do not lead to enhanced interactions with cells due to shielding by the external PEG chains. By varying the CPL and external PEG chain lengths, liposomal systems could be generated in which the CPL positive charge is shielded during transport in the circulation, becoming exposed only as the PEGCer exchanges out of the vesicles. Such systems may have utility for in vivo applications (see text for more details). It should be noted that the PEG chains are drawn as extended or globular for illustration purposes only, and are not intended to model actual chain conformations.

DSPE and oligosaccharide PEG-DSPE conjugates [20]. In these studies, between 1 and 3 mol% of conjugate (relative to total lipid) was incorporated into the target membranes. These values are significantly lower than the maximum insertion levels we report for the CPLs (up to 7 mol%). In addition, the oligopeptide/oligosaccharide PEG-DSPE conjugates could be inserted to 1.2 mol% into vesicles containing 3 mol% MPEG-DSPE, demonstrating the potential for using this approach with long-circulating lipo-

somes. In our systems, high insertion levels (up to 7 mol%) could be achieved for vesicles containing ~7 mol% $PEGCerC_{20}$. Although PEG-Cers will exchange from vesicles both in vitro [9] and during circulation [37,38], only a small portion of the $PEGCerC_{20}$ appears to be lost during CPL insertion (Table 1).

A recent study by Zalipsky and co-workers [14] reported on the incorporation of folic acid PEG-DSPE conjugates into vesicles, and demonstrated enhanced binding to cells expressing the folate receptor. The best binding was observed for vesicles containing only the folate PEG-DSPE conjugate. The presence of additional mPEG-DSPE greatly reduced binding, particularly when the length of the PEG in the two molecules was identical. Enhanced binding was only observed for vesicles containing folate-PEG_{3350}-DSPE and $mPEG_{2000}$-DSPE. This mirrors our system, where the M_rs of the PEG in the CPL and PEG-Cers are 3400 and 2000, respectively, and suggests we should be able to modulate cell binding by varying the lengths of the two PEG spacers (see below).

As shown in Fig. 6, cationic LUVs composed of $DOPE/DODAC/PEGCerC_{20}$ exhibit little uptake when incubated on BHK cells. Although positively charged vesicles exhibit enhanced binding to some cell lines, this can be attenuated by the presence of PEG on the liposome surface [21]. Clearly, for these systems, the presence of 6 mol% of positively charged DODAC leads to only low uptake levels after 6 h. Incorporation of 5.6 mol% of CPL_2 has little effect on uptake, which was only slightly improved in the presence of 5.7 mol% CPL_3. The best results were obtained with the CPL_{4b} (at 3.4 mol%), which possessed four positive charges. At 6 h incubation, a 10-fold increase in uptake was observed relative to the control vesicles. Several points can be surmised from these data. The first is that the presence of positively charged groups located some distance from the LUV surface can lead to significant increases in cellular uptake. In this case, the positive charges of the CPL (PEG $M_r = 3400$) are located above the surface coating of PEG ($M_r = 2000$), and thus are available for interactions with cells. However, it is not total charge alone that plays a role in enhanced cell binding. The quantity of positive charge present for the CPL_2 and CPL_{4b} samples is approximately equal, and yet the former shows little uptake compared to the latter. The CPL_3 sample has a greater total positive charge than the CPL_{4b} sample, and yet exhibits only a third of the uptake. It would appear that localization of a sufficient positive charge density at the distal end of the CPL molecule is an important parameter in ensuring interaction with cells. At least four charges seem to be required for efficient cell binding to occur.

The results of this paper demonstrate that the interaction of LUVs with cells in vitro can be significantly enhanced by increasing the positive charge present on the LUV surface by post-insertion of CPL. The additional positive charge must be physically distant from the surface to be available for interactions with cells, particularly when working with polymer-coated vesicles that are designed for minimal interactions with serum proteins and macrophages. This naturally leads to questions concerning the design of next generation liposomal systems capable of systemic drug or gene delivery following i.v. administration. First, it should be noted that the CPL systems described here are unlikely to be of direct utility as systemic delivery vectors. This is because the CPL employed contains a PEG_{3400} linker, and thus the cationic groups at the end of the PEG will extend beyond the PEG_{2000} 'cloud' provided by the PEG-Cer components of the LUVs. Charged liposomal systems are rapidly cleared from the circulation following i.v. administration, and it would therefore be expected that the systems employed here would not exhibit the long circulation lifetimes that lead to accumulation at disease sites such as tumors. Strategies that could overcome this difficulty include the possibility of inserting CPL containing a shorter PEG linker, such as PEG_{1000}. The positive charge should then be shielded by the PEG_{2000} of the PEG-Cer, leading to longer circulation lifetimes. If necessary, a greater degree of shielding could be achieved by insertion of the shorter-chain CPL into systems containing PEG-Cer molecules prepared using PEG_{5000}. A remaining problem concerns the need to achieve exposure of the CPL after arrival at the tumor site to stimulate uptake into cells. As we have noted elsewhere [9,36,37], the PEG-Cer component of liposomal systems can be designed to dissociate over a time determined by the length of the acyl chain contained in the ceramide anchor. If the

PEG-Cer is designed to dissociate slowly, a situation can be envisioned where LUV that have accumulated at a disease site such as a tumor will lose the PEG shield, thus exposing the CPL which stimulate uptake. An alternative possibility is to post-insert lipids containing specific targeting ligands, that do not stimulate clearance, at the end of the PEG linker. Optimizing these systems for in vivo use will thus involve varying the chain length of the PEG polymers of both CPL and PEGCer, and of the fatty acyl moieties which determine PEGCer exchange rates.

In summary, the results presented here demonstrate the insertion of a new class of cationic ligands into preformed liposomal systems, leading to increased uptake into cells. The approach should be generally applicable to liposomes containing small molecule drugs (e.g., anticancer drugs) as well as liposomes containing macromolecular drugs, such as plasmids containing genes coding for therapeutic proteins. Current studies are focused on applying this approach to a new class of lipid-based DNA carrier systems known as stabilized plasmid–lipid particles (SPLPs) [9].

Acknowledgements

This research was supported by the Medical Research Council of Canada.

References

[1] P.R. Cullis, M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer, D.B. Fenske, Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles, Biochim. Biophys. Acta 1331 (1997) 187–211.

[2] L.D. Mayer, L.C. Tai, D.S. Ko, D. Masin, R.S. Ginsberg, P.R. Cullis, M.B. Bally, Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice, Cancer Res. 49 (1989) 5922–5930.

[3] L.D. Mayer, M.B. Bally, P.R. Cullis, S.L. Wilson, J.T. Emerman, Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor, Cancer Lett. 53 (1990) 183–190.

[4] N.L. Boman, L.D. Mayer, P.R. Cullis, Optimization of the retention properties of vincristine in liposomal systems, Biochim. Biophys. Acta 1152 (1993) 253–258.

[5] N.L. Boman, D. Masin, L.D. Mayer, P.R. Cullis, M.B. Bally, Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors, Cancer Res. 54 (1994) 2830–2833.

[6] N.L. Boman, V.A. Tron, M.B. Bally, P.R. Cullis, Vincristine-induced dermal toxicity is significantly reduced when the drug is given in liposomes, Cancer Chemother. Pharmacol. 37 (1996) 351–355.

[7] P.L. Felgner, G.M. Ringold, Cationic liposome-mediated transfection, Nature 337 (1989) 387–388.

[8] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.

[9] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, Y.P. Zhang, M.J. Hope, P. Scherrer, P.R. Cullis, Stabilized plasmid-lipid particles: construction and characterization, Gene Ther. 6 (1999) 271–281.

[10] K.W. Mok, A.M. Lam, P.R. Cullis, Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties, Biochim. Biophys. Acta 1419 (1999) 137–150.

[11] O. Meyer, D. Kirpotin, K. Hong, B. Sternberg, J.W. Park, M.C. Woodle, D. Papahadjopoulos, Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides, J. Biol. Chem. 273 (1998) 15621–15627.

[12] G.Y. Kao, L.J. Change, T.M. Allen, Use of targeted cationic liposomes in enhanced DNA delivery to cancer cells, Cancer Gene Ther. 3 (1996) 250–256.

[13] C.B. Hansen, G.Y. Kao, E.H. Moase, S. Zalipsky, T.M. Allen, Attachment of antibodies to sterically-stabilized liposomes: evaluation, comparison and optimization of coupling procedures, Biochim. Biophys. Acta 1239 (1995) 133–144.

[14] A. Gabizon, A.T. Horowitz, D. Goren, D. Tzemach, F. Mandelbaum-Shavit, M.M. Qazen, S. Zalipsky, Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: In vitro studies, Bioconjug. Chem. 10 (1999) 289–298.

[15] R.J. Lee, P.S. Low, Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis, J. Biol. Chem. 269 (1994) 3198–3204.

[16] J.A. Reddy, P.S. Low, Folate-mediated targeting of therapeutic and imaging agents to cancers, Crit. Rev. Ther. Drug Carrier Syst. 15 (1998) 587–627.

[17] S.R. Holladay, Z. Yang, M.D. Kennedy, C.P. Leamon, R.J. Lee, M. Jayamani, P.S. Mason, Low, Riboflavin-mediated delivery of a macromolecule into cultured human cells, Biochim. Biophys. Acta 1426 (1999) 195–204.

[18] S. Wang, P.S. Low, Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells, J. Controlled Release 53 (1998) 39–48.

[19] S. Zalipsky, B. Puntambekar, P. Boulikas, C.M. Engbers, M.C. Woodle, Peptide attachment to extremities of lipo-

somal surface grafted PEG chains: preparation of the long-circulating form of laminin pentapeptide, YIGSR, Bioconjug. Chem. 6 (1995) 705–708.
[20] S. Zalipsky, N. Mullah, J.A. Harding, J. Gittelman, L. Guo, S.A. DeFrees, Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains, Bioconjug. Chem. 8 (1997) 111–118.
[21] C.R. Miller, B. Bondurant, S.D. McLean, K.A. McGovern, D.F. O'Brien, Liposome-cell interactions in vitro: effect of liposome surface charge on the binding and endocytosis of conventional and sterically-stabilized liposomes, Biochemistry 37 (1998) 12875–12883.
[22] I.M. Hafez, S. Ansell, P.R. Cullis, Tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipids, Biophys. J. 79 (2000) 1438–1446.
[23] T. Chen, K.F. Wong, D.B. Fenske, L.R. Palmer, P.R. Cullis, Fluorescent-labeled poly(ethylene glycol) lipid conjugates with distal cationic headgroups, Bioconjug. Chem. 11 (2000) 433–437.
[24] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential., Biochim. Biophys. Acta 812 (1985) 55–65.
[25] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 858 (1986) 161–168.
[26] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[27] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[28] J.R. Silvius, R. Leventis, Spontaneous interbilayer transfer of phospholipids: dependence on acyl chain composition, Biochemistry 32 (1993) 13318–13326.
[29] J.R. Silvius, M.J. Zuckermann, Interbilayer transfer of phospholipid-anchored macromolecules via monomer diffusion, Biochemistry 32 (1993) 3153–3161.
[30] P.S. Uster, T.M. Allen, B.E. Daniel, C.J. Mendez, M.S. Newman, G.Z. Zhu, Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time, FEBS Lett. 386 (1996) 243–246.
[31] T.M. Allen, G.A. Austin, A. Chonn, L. Lin, K.C. Lee, Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size, Biochim. Biophys. Acta 1061 (1991) 56–64.
[32] K.D. Lee, S. Nir, D. Papahadjopoulos, Quantitative analysis of liposome-cell interactions in vitro: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes, Biochemistry 32 (1993) 889–899.
[33] K.D. Lee, K. Hong, D. Papahadjopoulos, Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density, Biochim. Biophys. Acta 1103 (1992) 185–197.
[34] P.L. Felgner, Y.J. Tsai, L. Sukhu, C.J. Wheeler, M. Manthorpe, J. Marshall, S.H. Cheng, Improved cationic lipid formulations for in vivo gene therapy, Ann. N. Y. Acad. Sci. 772 (1995) 126–139.
[35] W.R. Jarnagin, R.J. Debs, S.S. Wang, D.M. Bissell, Cationic lipid-mediated transfection of liver cells in primary culture, Nucleic Acids Res. 20 (1992) 4205–4211.
[36] K. Sou, T. Endo, S. Takeoka, E. Tsuchida, Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles, Bioconjug. Chem. 11 (2000) 372–379.
[37] M.S. Webb, D. Saxon, F.M.P. Wong, H.J. Lim, Z. Wang, M.B. Bally, L.S.L. Choi, P.R. Cullis, L.D. Mayer, Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol) – effects on the pharmacokinetics of liposomal vincristine, Biochim. Biophys. Acta 1372 (1998) 272–282.
[38] M.A. Monck, A. Mori, D. Lee, P. Tam, J.J. Wheeler, P.R. Cullis, P. Scherrer, Stabilized plasmid-lipid particles: pharmacokinetics and plasmid delivery to distal tumors following intravenous injection, J. Drug Target. 7 (2000) 439–452n.